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(57) Abstract

An invention is described that permits the regulation of recombination in cells, organisms or appropriate cell-free systems. The invention involves creating fusion proteins between recombinase proteins, or components of recombinase systems, and ligand binding domains derived from nuclear receptors. The fusion proteins show little recombinase activity in the absence of the ligand that binds to the ligand binding domain. Upon binding of the ligand, recombinase activity is induced. The invention provides a practical means to regulate recombination in cells and organisms and, by linking ligand binding to recombination, provides a simple means whereby ligand binding can be measured as recombination achieved.

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Regulation of site-specific recombination by site-specific recombinase/nuclear receptor fusion proteins

Specification

The present invention relates to the technical field of genetic manipulation, and more specifically, to the use of recombination-mediated DNA rearrangements and to the use of the regulatory potential of nuclear receptors.

The use of site-specific recombinases (SSRs) to induce defined rearrangements of DNA has been described in a variety of organisms (1-12). These reports describe the introduction of a DNA construct that contains SSR target sites. Subsequent exposure to the SSR enzyme activity resulted in the DNA rearrangement determined by the disposition of the target sites (see reference 13 for a recent review of SSRs). Three SSRs have been used in this manner to date; FLP recombinase from the 2μ episome of Saccharomyces cerevisiae (1,2,5,6,9,10), CRE recombinase from the Escherichia coli phage P1 (3,4,8,11,12) and R recombinase from pSR1 of Zygosaccharomyces rouxii (7). Amongst other SSR systems relevant to the invention described here are those listed in references 13 and 14, and SSRs from Kluyveromyces drosophilarium (15), Kluyveromyces waltii (16), λ Int (17) and the Gin recombination system from phage Mu (18). The content of the above document is incorporated by reference.

For many applications in cells, organisms and cell-free in vitro systems, SSR induced DNA rearrangements must be regulated. Current implementation of the potential offered by SSRs is limited by the means available to regulate SSR activity. In experiments with cultured cells, unregulated SSRs have been used. For example, after introduction of SSR target sites into cells, recombination has been induced by subsequent introduction of either FLP recombinase by transfection of DNA (4,9) or injection of CRE recombinase protein (12). That is, the intended recombination event was regulated merely by the time of introduction of an appropriate macromolecule. Amongst other limitations, this precludes the creation and proliferation of homogeneous populations of cells that contain both the unrear-

ranged target sites and the SSR and in which the recombination event can be induced after cell numbers have been expanded.

In experiments with transgenic animals, the issue of SSR regulation has been addressed by regulating the expression of an introduced SSR gene using the inducible heat-shock promoter in Drosophila (5) or a tissue-specific promoter in mice (11). Both approaches have limited applicability. Namely, heat-shock regulation of transgene expression is currently only useful in flies and no suitable counterpart is available for use in cell lines or vertebrates. Also, the use of a tissue-specific promoter to regulate transgene expression relies on the limited availability of suitable promoters and enhancers and the expression pattern achieved is confined to the times and places at which these tissue specific elements are active.

The problem underlying the present invention was to provide a regulated recombination system, wherein the disadvantages of the prior art are at least partially eliminated. More specifically, the problem was to provide a recombination system, wherein the recombination event can be induced independently from any tissue specific restrictions.

This patent application describes an invention that regulates SSR activity, rather than its expression. Thus any means of achieving and directing expression can be used, such as using ubiquitous or broadly active promoters and enhancers, as well as tissue specific or inducible promoters and enhancers.

One aspect of the present invention relates to a fusion protein, comprising a recombinase protein or a component of a recombinase complex, fused to part or all of a nuclear receptor, so that the amino acids that bind the ligand of said nuclear receptor are included, such that in cells or appropriate cell-free systems: (a) recombinase activity is inhibited in the absence of ligand binding to said ligand binding domain and (b) recombinase activity is induced or altered by binding of ligand to said ligand binding domain.

Preferably the recombinase activity is altered by binding of ligand to ligand binding domain by a factor of at least 10, more preferably of at least 20 and most preferably of at least 40.

The present invention resides in the regulation of SSR activity by fusing an SSR protein to the ligand binding domain (LBD) of a nuclear receptor. SSR-LBD fusion proteins comprise an amino acid sequence of SSRs physically attached to the amino acid sequence of an LBD of a nuclear receptor. That is, the invention is a fusion protein, comprising a recombinase protein, or a component of a recombinase complex, fused to the ligand binding domain of a nuclear receptor. Preferably, the recombinase protein or a component of a recombinase complex is fused to the nuclear receptor or ligand binding domain thereof by means of genetic fusion, i.e. the SSR-LBD protein is a linear genetic fusion encoded by a single nucleic acid. On the other hand, the present invention also encompasses SSR-LBD fusion proteins which are linked by different means, e.g. through a spacer molecule having reactive groups thereon, which are covalently bound to each protein domain.

Most simply, the attachment of the SSR and LBD components can be achieved by making a DNA construct that encodes for the amino acid sequence of the SSR-LBD fusion protein with the LBD encoding DNA placed in the same reading frame as the SSR encoding DNA, preferably either at the amino or carboxy termini of the SSR protein (19). More preferably, the nuclear receptor or ligand binding domain thereof is fused to the C-terminus of the recombinase protein or component of a recombinase complex. In an especially preferred embodiment of the present invention the nuclear receptor or ligand binding domain thereof is fused to the recombinase protein or component of a recombinase complex through a peptide linker which mainly consists of hydrophilic acids and preferably has a length of 4 to 20 amino acids.

SSR-LBD fusion proteins can coexist with target sites without recombination occurring since these proteins require ligand binding to the LBD for recombinase activity. In our experi-

ments, recombinase activity of the SSR-LBD fusion protein, in the absence of the relevant ligand, is at least 200x less active than wild type recombinase activity. Upon presenting the SSR-LBD fusion proteins with the relevant ligand, recombinase activity is induced to more than 20% of wild type, that is, equal to or greater than 40x induction. This is the first description of post-transcriptional regulation of SSRs by any means. In particular, the invention permits the propagation of both the unrearranged target sites and the SSR-LBD in living systems. More specifically:

(a) cell lines can be established that carry both the unrearranged target sites and the SSR-LBD as a prerequisite for biochemical studies or introduction into organisms. The cell lines can be homogeneous and characterised before expansion to the required quantities and subsequent induction of recombination by administration of the relevant ligand,

(b) recombination can be regulated in any experimentally manipulatable multicellular organism by administration of the relevant ligand. The SSR-LBD could be introduced to the organism either through the incorporation of cells or by direct means such as microinjection or in the genome of a viral based vector. Recombination can be induced after characterisation by administration of the relevant ligand.

The term "nuclear receptor", as used herein, refers to a molecule, preferably a protein molecule, which may be glycosylated or unglycosylated, having the abilities to bind ligand and to be incorporated into a nucleus of a cell. Specifically, the term nuclear receptor refers to those proteins that display functional or biochemical properties that are similar to the functional or biochemical properties displayed by the steroid hormone receptors with respect to ligand binding, for example, the dioxin receptor (Whitelaw, M.L., Göttlicher, M., Gustafsson, J.-A. and Poellinger, L., (1993) EMBO J. 12, 4169-4179). More specifically, the term nuclear receptor refers to those proteins that are related by their amino acid sequence to the LBDs of the steroid hormone receptors. The paper of Laudet et al. (1992) (Laudet, V., Hänni, C., Coll, J., Catzeflis, F. and Stehelin, D. (1992) EMBO J. 11, 1003-1013) describes an align-

ment of nuclear receptor amino acid sequences using standard methodologies. Included in the definition of nuclear receptors used here are also those proteins not listed in Laudet et al. but which can be included using the methods employed by Laudet et al.. The term nuclear receptor also includes mutant derivatives of nuclear receptor amino acid sequences which retain sufficient relatedness to nuclear receptor amino acid sequences as to be identifiable as related using the methods employed by Laudet et al.

The nuclear receptor which is fused to the recombinase protein is preferably a hormone receptor, e.g. a receptor recognized by steroids, vitamins or related ligands. Examples of suitable nuclear receptors are listed in reference 20, which is hereby incorporated by reference. Preferably, the nuclear receptor is a steroid hormone receptor, more preferably, a glucocorticoid, estrogen, progesteron, or androgen receptor.

In the SSR-LBD fusion protein of the present invention, it is not required that the complete nuclear receptor is present; i.e. it is sufficient that the amino acids that bind the ligand are fused to the SSR.

Upon binding their relevant ligand, nuclear receptors become active, or altered, transcription factors. The cloning of cDNAs encoding members of the steroid receptor family of proteins revealed that they share amino acid sequence homology, in particular in the protein domain that binds ligand. The LBD can be separated from the rest of the protein and fused to other transcription factors conferring ligand regulation onto the resulting fusion proteins. For the glucocorticoid and estrogen receptors, the domain that binds ligand has been fused to other transcription factors and also to oncoproteins, rendering the fusion proteins dependent on the relevant ligand for activity (19,21-26). All characterised oncoproteins that have been experimentally regulated in this manner so far are also transcription factors. Transcription factors and oncoproteins are not enzymes in the classical sense and the regulation of their activities by steroids and LBDs described in references 19, 21-

26, is essentially similar to the role that LBDs play in regulating steroid receptors. Prior attempts to extend the regulatory possibilities of the LBD fusion protein strategy beyond transcription factors and oncoproteins have yet to be reported. Attempts to regulate the enzyme activities of either ß-galactosidase or dihydrofolate reductase by fusing them with the glucocorticoid LBD have been unsuccessful (D. Picard, public seminar given at EMBL, Heidelberg, Nov. 1991).

The present invention extends the regulatory possibilities of the LBD fusion protein strategy beyond the currently documented transference of ligand regulation from nuclear receptors to other transcription factors, to include an enzyme activity, namely a site specific recombinase. The term "site specific recombinase" refers to any protein component of any recombinant system that mediates DNA rearrangements in a specific DNA locus, including site specific recombinases of the integrase or resolvase/invertase classes (13; Abremski, K.E. and Hoess, R.H. (1992) Protein Engineering 5, 87-91; Khan, E., Mack, J.P.G., Katz, R.A., Kulkosky, J. and Skalka, A.M. (1991) Nucleic acids Res. 19, 851-860) and site-specific recombination mediated by intron-encoded endonucleases (Perrin, A., Buckle, M. and Dujon, B. (1993) EMBO J. 12, 2939-2947). Preferred recombinase proteins, which can be used in the fusion proteins according to the invention, are selected from the group consisting of: FLP recombinase, Cre recombinase, R recombinase from the Zygosaccharomyces rouxii plasmid pSR1, A recombinase from the Kluyveromyces drosophilarium plasmid pKD1, A recombinase from the Kluyveromyces waltii plasmid pKW1, any component of the λ Int recombination system, any component of the Gin recombination system, or variants thereof. The term "variant" in this context refers to proteins which are derived from the above proteins by deletion, substitution and/or addition of amino acids and which retain their some or all of the function inherent in the protein from which they are derived. Specifically, the variant could retain the ability to act as a recombinase, or it could retain protein/protein or protein/DNA interactions critical to the recombination reaction, or to the regulation of the recombination reaction.

In a preferred embodiment of the invention, FLP recombinase is fused to the LBD of the estrogen, glucocorticoid, progesterone or androgen receptors (20). Other preferred embodiments include fusing Cre recombinase or R recombinase, or SSRs from Kluyveromyces drosophilarium or Kluyveromyces waltii, to these LBDs. Another preferred embodiment involves regulating one or more components of an SSR complex to these LBDs, in particular, components of the λ Int or Gin recombination systems. The invention, in providing a means to regulate recombination, is however not limited to known recombinases and recombination complexes and is not limited to known nuclear receptor LBDs. Rather, the strategy of fusing recombinases, or components of recombination complexes, to LBDs of nuclear receptors is applicable to any fusion combination of these proteins which display the desired characteristics readily identifiable without undue experimentation on the part of a skilled person.

A further subject-matter of the present invention is a nucleic acid which encodes the fusion protein according to the present invention. Preferably, the nucleic acid is a DNA or RNA.

Still a further subject-matter of the present invention is a recombinant vector comprising at least one copy of the nucleic acid as defined above. This recombinant vector may be a eukaryotic vector, a viral vector, or a prokaryotic vector, or a vector which can be maintained in eukaryotic and prokaryotic host cells. The recombinant vector is obtainable by inserting a nucleic acid encoding a SSR-LBD fusion protein into a suitable starting vector. Specific examples of suitable starting vectors are given for example in Molecular Cloning. A Laboratory Manual, 2nd edition, J. Sambrook et al. (1989), Cold Spring Harbor Laboratory Press, chapters 1, 2, 3, 4, 16 and 17.

The vector of the present invention may be an extra-chromosomal vector or a vector which integrates into the genome of the host cell. Extra-chromosomal vectors, e.g. circular plasmids are especially preferred. An example of a plasmid vector is the plasmid pHFE1 comprising a nucleic acid encoding a fusion of the FLP recombinase and the ligand binding domain of the estro-

gen receptor. The plasmid pHFE1 has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Mascheroder Weg 1b, D-38124 Braunschweig on June 25, 1994 under the accession number DSM 9265 according to the requirements of the Budapest Treaty.

Still a further subject-matter of the present invention is a cell containing a nucleic acid or a recombinant vector as defined above. Preferably, the cell is stably transformed with the nucleic acid or the vector. Suitable cells are eukaryotic or prokaryotic cells. Examples of prokaryotic cells are gram-negative bacterial cells, especially E.coli cells. Examples of eukaryotic cells are mammalian cells, yeasts and trypanosomes.

The invention separates the function of ligand binding from the functions of transcription factors, coupling ligand binding to recombinase activity. Thereby, the binding of ligand can be assessed by any means that measures recombinase activity. Thus, further embodiments of the invention include methods for determining the binding of ligand to the LBD of a nuclear receptor, comprising the steps of:
(i) the introduction of the SSR-LBD fusion protein or the nucleic acid coding therefor into cells, or appropriate cell-free systems, that contain the DNA target sites for the SSR; (ii) administering the ligand or a mixture suspected to contain a ligand or ligands to be evaluated; if the ligand is not already present in the system; (iii) detecting the recombinase activity, if any, of the SSR-LBD by detecting, directly or indirectly, recombination or changes in the recombination rate between the DNA target sites.

The introduction of SSR-LBD fusion proteins into cells may be accomplished by transforming the cells with appropriate vectors containing a nucleic acid coding for the SSR-LBD fusion protein. The administration of the ligand to be evaluated is not necessary in systems which already contain the ligand and in which the ligand-concentration is determined by detecting recombination between DNA target sites.

Particularly preferred embodiments include:
(a) direct measurements of the physical arrangement of the target sites using techniques such as gel electrophoresis of DNA molecules, Southern blotting or polymerase chain reaction.
(b) indirect measurements by assessing the properties encoded by the DNA regions carrying the target sites before or after recombination. For example, recombination could excise an antibiotic resistance gene from the genome of the host and thus recombination can be measured as sensitivity to the antibiotic.
(c) the measurement of intracellular ligand concentrations by evaluating recombination between the DNA target sites resident in the cells that also carry the SSR-LBD fusion proteins.
(d) the evaluation of ligand binding to an LBD without the need to use radiolabelled ligand derivatives or without reliance on the transcription factor properties of the corresponding nuclear receptor.
(e) evaluating the effect of mutations in the LBD on ligand binding. Since SSR-LBD recombination requires ligand, mutations in the LBD that decrease ligand binding can be ascertained. Alternatively, mutations that improve binding of a different ligand can be selected. For example, ligand-dependent recombination could rearrange the DNA, so that an antibiotic resistance gene is expressed. Cells with the rearranged DNA will grow under the appropriate antibiotic selection, cells with unrearranged DNA will not. Specifically this describes a method for determining the effect of mutations in the LBD of a nuclear receptor on its ability to bind ligand, comprising the steps of (a) introducing mutations into the LBD of the SSR-LBD fusion protein, and (b) following steps (i) to (iii) above.

The invention also encompasses a method for regulating the recombination of DNA target sites, comprising the steps of:
(a) providing cells or appropriate cell-free systems that contain DNA target sites for a site-specific recombinase,
(b) contacting the DNA target sites with a fusion protein according to the present invention, which contacting may be accomplished by direct introduction of the protein or by transformation of a cell with the nucleic acid encoding the protein and expressing the nucleic acid to produce the protein,

(c) effecting a recombination of said DNA target sites by contacting the fusion protein with a ligand for the nuclear receptor component of the fusion protein, e.g. by adding the ligand to the system at a predetermined time, or by effecting production of the ligand in the system at a predetermined time.

Description of the Figures

Figure 1A depicts the plasmid, pNEOßGAL (thin line), integrated into the genome of 293 cells (thick line). FLP recombinase target sites (FRTs) are depicted as broad arrows and the neomycin resistance gene lies between the two FRTs. Upon estradiol administration, the DNA between the FRTs is excised, leaving one FRT in the genome and one on the excised circular DNA. The probe used for the Southern blot of Fig. 2 is also depicted.

Figure 1B depicts the steps involved in the creation of plasmid pHFE1.

Figure 2 shows a Southern blot of a timecourse of recombination occurring in the cell line P1.4. At the times indicated after estradiol, or ethanol only, administration in the upper part of the figure, cells were lysed, DNA purified, restricted with NdeI, run on a 1.25% agarose gel and blotted to Biodyne B membranes by standard methodologies. The membrane was hybridized with the radioactively labelled probe depicted in Fig. 1A. The upper band shows the unrecombined integrated DNA and the lower the recombined band. Estradiol was dissolved in ethanol and the extreme right hand lane shows cells treated with the equivalent amount of ethanol, without estradiol, for 51 hours.

Figure 3 shows the complete amino acid sequence of the SSR-LBD-fusion polypeptide encoded by the plasmid pHFE1. The FLP recombinase domain is located from amino acid 1-423. The linker peptide is located from amino acid 424-428. The estrogen binding domain is located from amino acid 429-773.

Figure 4 shows the nucleic acid sequence coding for the SSR-LBD fusion polypeptide of Figure 3. The parts coding for the FLP

recombinase domain, the linker peptide and the estrogen binding domain, respectively, are specified.

The following examples are offered by way of illustration and is not intended to limit the invention in any manner.

Example 1

Construction of plasmid containing a SSR-LBD-fusion gene

The experiment involves two plasmids, one of which is the target for recombination, and the other is the recombinase expression plasmid. As the target plasmid, pNEOßGAL (9) was employed. pNEOßGAL contains two FLP recombinase target sites (FRT) surrounding a gene encoding neomycin resistance (Fig. 1a). The expression plasmid, pHFE 1, carries two genes, one encoding an FLP recombinase-estrogen receptor LBD fusion protein and the other encoding hygromycin resistance (Fig. 1b). The plasmid was constructed by standard cloning procedures using pBluescribe (Stratagene), pOG 44 (9), pHE63 (22) and pCNH2 (27). The stop codon of the FLP recombinase coding region present in p0G44 was mutated by oligonucleotide replacement to introduce BamH1, BsiW1 and EcoR1 sites and continue the open reading frame. The BamHI site in the estrogen receptor encoded by pHE63 was used to join the coding regions of FLP recombinase and estrogen receptor LBD. All of the estrogen receptor coding region carboxy to the BamHI site is present in the plasmid, including its stop codon.

Example 2

Regulation of site specific recombination

pNEOßGAL was introduced into 293 human embryonal kidney cells by electroporation (5 x 10^6 cells in 500 μl phosphate-buffered saline with 5 μg of ApaI linearised plasmid; 300 V at 125 μF in a 1 ml cuvette using a Bio-Rad Gene Pulser) and selection for neomycin resistance (G418, 0.4 mg/ml). Single colonies of resistant cells were cloned and characterised for incorporation of target plasmid DNA by Southern blotting. A clone, P1, showing integration of a single copy of target plasmid DNA was selected and transfected, using the calcium phosphate procedure, with

the second plasmid, pHFE 1 (1 μg of XmnI linearised pHFE 1 was precipitated by the standard calcium phosphate method onto 10^7 P1 cells which were cultured in DMEM without phenol red, 10% charcoal stripped fetal calf serum). Four clones resistant to both neomycin and hygromycin (0.4 mg/ml G418, 0.4 mg/ml hygromycin B) were isolated and the dependance of recombination on estradiol administration was observed in all four. For clone P1.4, a time course of recombination in the presence of 10^{-6} M estradiol is shown (Fig. 2).

References

1. Falco,S.C., Li,Y., Broach,J.R. and Botstein,D. (1982) Cell 29, 573-584.
2. Cox,M.M. (1983) Proc. Natl. Acad. Sci. USA 80, 4223-4227.
3. Sauer,B. (1987) Mol. Cell. Biol. 7, 2087-2096.
4, Sauer,B. and Henderson,N. (1989) Nucleic Acids Res. 17,147-161.
5. Golic,K.G. and Lindquist,S. (1989) Cell 59, 499-509.
6. Cregg,J.M. and Madden,K.R. (1989) Mol. Gen. Genet. 219, 320-323.
7. Matsuzaki,H. et al, (1990) J. Bacteriol. 172, 610-618.
8. Odell,J., Caimi,P., Sauer,B. and Russell,S. (1990) Mol. Gen. Genet. 223, 369-378.
9. O'Gorman,S., Fox,D.T. and Wahl,G.M. (1991) Science 251, 1351-1355.
10. Konsolaki,M. et al (1992) New Biologist 4, 551-557.
11. Lakso,M. et al (1992).Proc. Natl. Acad. Sci. USA 89, 6232-6236.
12. Baubonis,W. and Sauer,B. (1993) Nucleic Acids Res. 21,2025-2029.
13. Stark,W.M. et al (1992) Trends Genet. 8, 432-439.
14. Utatsu,I. et al (1987) J. Bacteriol. 169, 5537-5545.
15. Chen,X.J. et al (1986) Nucleic Acids Res. 14, 4471-4481.
16. Chen,X.J. et al (1992) J. Gen. Microbiol. 138, 337-345.
17. Landy,A. (1989) Annu. Rev. Biochem. 58, 913-949.
18. Klippel,A. et al (1993) EMBO J. 12, 1047-1057.

19. Webster,N.J.G. et al (1988) Cell 54, 194-207

20. Beato,M. (1989) Cell 56, 335-344.

21. Kumar,V. et al (1987) Cell 51, 941-951.

22. Picard,D., Salser,S.J. and Yamamoto,K.R. (1988) Cell 54, 1073-1080.

23. Eilers,M., Picard,D., Yamamoto,K.R. and Bishop,M. (1989) Nature 340, 66-68.

24. Superti-Furga,G. et al (1991) Proc. Natl. Acad. Sci. USA 88, 5114-5118.

25. Burk,O. and Klempenauer,K.-H. (1991) EMBO J. 10, 3713-3719.

26. Boehmelt,G. et al (1992) EMBO J. 11, 4641-4652.

27. Schmidt,E. et al (1990) Mol. Cell. Biol. 10, 4406-4411.

Claims

1. A fusion protein, comprising a recombinase protein or a component of a recombinase complex, fused to part or all of a nuclear receptor so that the amino acids that bind the ligand of said nuclear receptor are included, such that, in cells or appropriate cell-free systems:
(a) recombinase activity is inhibited in the absence of ligand binding to said ligand binding domain, and
(b) recombinase activity is induced or altered by binding of ligand to said ligand binding domain.

2. The fusion protein of claim 1, wherein the nuclear receptor is a hormone receptor.

3. The fusion protein of claim 1 or 2, wherein the nuclear receptor is a steroid hormone receptor.

4. The fusion protein of any one of the claims 1-3, wherein the nuclear receptor is a mutated derivative of a nuclear receptor such that it retains the characteristics of the fusion protein of claim 1.

5. The fusion protein of any of the claims 1 - 4, wherein the nuclear receptor is a vertebrate glucocorticoid, estrogen, progesteron or androgen receptor.

6. The fusion protein of any one of the claims 1-5, wherein the recombinase protein or component of a recombinase complex is selected from the group consisting of: FLP recombinase, Cre recombinase, R recombinase from the Zygosaccharomyces rouxii plasmid pSR1, A recombinase from the Kluyveromyces drosophilarium plasmid pKD1, A recombinase from the Kluyveromyces waltii plasmid pKW1, any component of the λ Int recombination system, any component of the Gin recombination system, or a variant thereof.

7. The fusion protein of any of claims 1-6,

wherein the recombinase protein or compound of a recombinase complex is fused to the nuclear receptor or ligand binding domain thereof by means of a genetic fusion.

8. The fusion protein of any of claims 1-7, wherein said nuclear receptor or ligand binding domain thereof is fused to the N- or C-terminus of said recombinase protein or component of a recombinase complex.

9. The fusion protein of any of claims 1-8, wherein said nuclear receptor is fused to said recombinase protein or component of a recombinase complex through a peptide linker.

10. The fusion protein of any of claims 1-9, which comprises the FLP recombinase and the ligand binding domain of the estrogen receptor.

11. A nucleic acid which encodes the fusion protein of any one of the claims 1-10.

12. The nucleic acid of claim 11, which is a DNA or RNA.

13. A recombinant vector comprising at least one copy of the nucleic acid of claims 11 or 12.

14. The vector of claim 13, which is a plasmid.

15. The plasmid pHFE1 (DSM 9265)

16. A cell containing a nucleic acid of claims 11 or 12 or a recombinant vector of any one of the claims 13-15.

17. The cell of claim 16, which is a mammalian cell.

18. A method for determining the binding of a ligand to the ligand binding domain of a nuclear receptor, comprising the steps of:

(a) the introduction of the fusion protein of claims 1-9, or the nucleic acid of claims 10 or 11 into cells, or appropriate cell-free systems, that contain the DNA target sites for recombination,
(b) optionally administering the ligand or a mixture suspected to contain a ligand or ligands, to be evaluated,
(c) detecting the recombinase activity, if any, of said fusion protein by detecting recombination or changes in the recombination rate between said DNA target sites.

19. The method of claim 18, wherein the recombination between said DNA target sites is detected by direct measurement of the physical arrangement of said target sites.

20. The method of claim 18, wherein the recombination between said DNA target sites is detected by assessing the properties encoded by the DNA regions carrying the target sites before or after recombination.

21. A method for determining the effect of mutations in the ligand binding domain of a nuclear receptor on its ability to bind ligand, comprising the steps of:
(a) introducing mutations into the ligand binding domain of the fusion protein of claims 1-10,
(b) following steps (a) to (c) of claim 18.

22. A method for regulating the recombination of DNA target sites, comprising the steps of:
(a) providing cells or appropriate cell-free systems that contain DNA target sites for a site specific recombinase,
(b) contacting the DNA target sites with a fusion protein according to any one of the claims 1-10, and
(c) effecting recombination of said DNA target site by contacting the fusion protein with a ligand for the nuclear receptor component of said fusion protein.

FIG. 1A

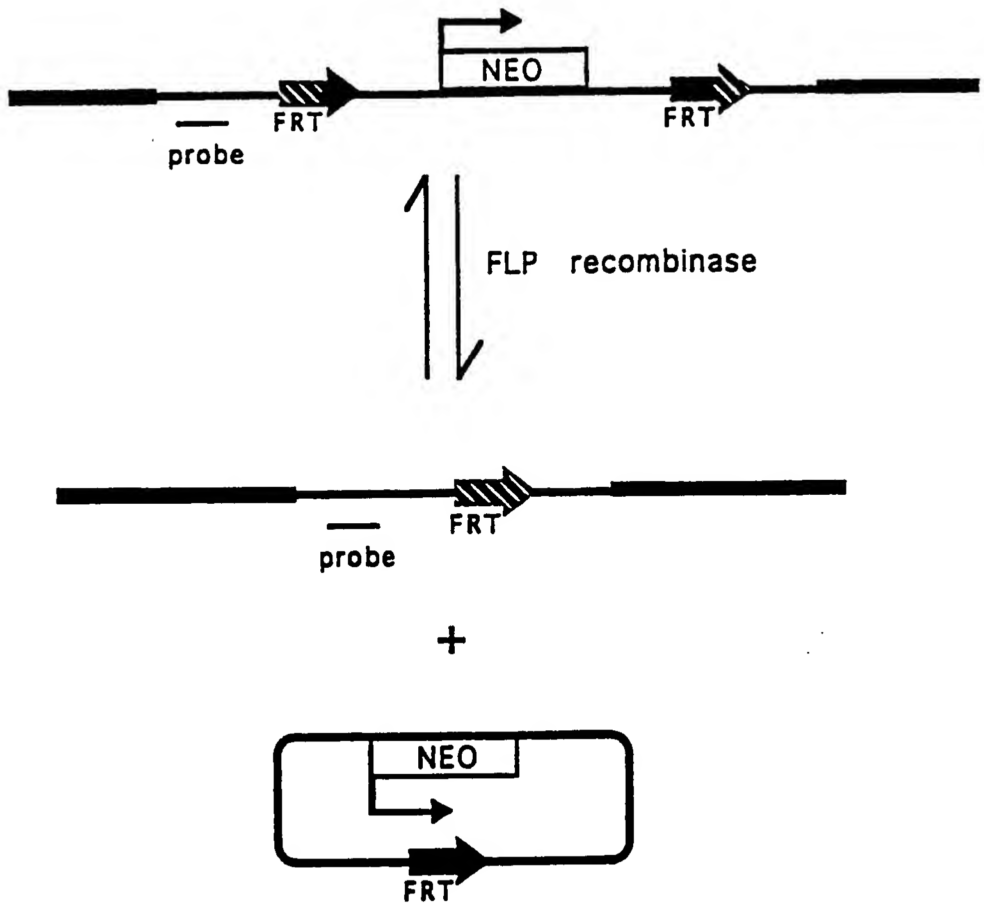

FIG. 1B

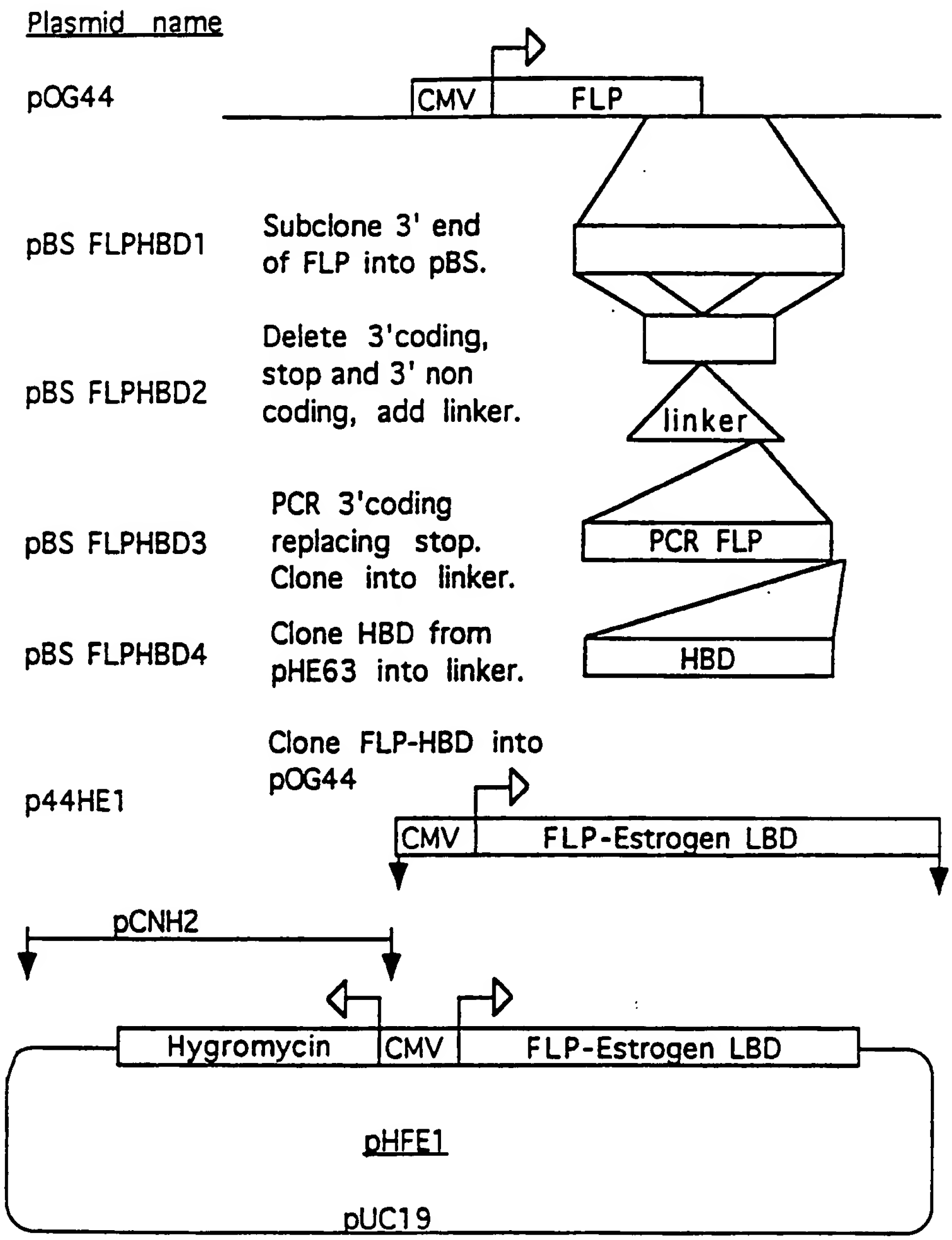
Plasmid name
pOG44
CMV
FLP
pBS FLPHBD1
Subclone 3' end of FLP into pBS.
pBS FLPHBD2
Delete 3'coding, stop and 3' non coding, add linker.
linker
pBS FLPHBD3
PCR 3'coding replacing stop. Clone into linker.
PCR FLP
pBS FLPHBD4
Clone HBD from pHE63 into linker.
HBD
p44HE1
Clone FLP-HBD into pOG44
CMV
FLP-Estrogen LBD
pCNH2
Hygromycin
CMV
FLP-Estrogen LBD
pHFE1
pUC19

FIG. 2

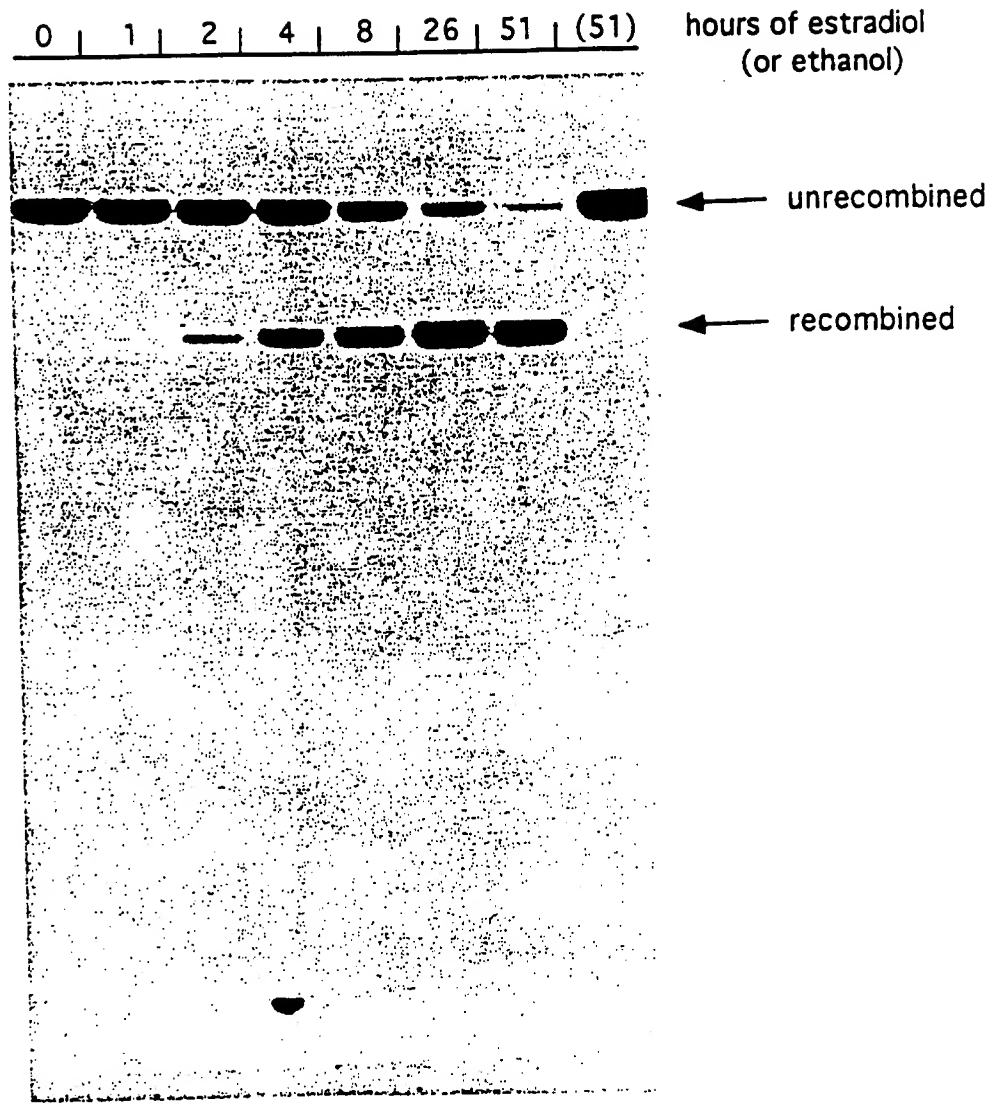
0
1
2
4
8
26
51
(51)
hours of estradiol (or ethanol)
unrecombined
recombined

Fig. 3

FLP/EBD AMINO ACID SEQUENCE AS ENCODED BY PHFE1

1 MPQFDILCKT PPKVLVRQFV ERFERPSGEK IALCAAELTY LCWMITHNGT

51 AIKRATFMSY NTIISNSLSL DIVNKSLQFK YKTQKATILE ASLKKLIPAW

101 EFTIIPYYGQ KHQSDITDIV SSLQLQFESS EEADKGNSHS KKMLKALLSE

151 GESIWEITEK ILNSFEYTSR FTKTKTLYQF LFLATFINCG RFSDIKNVDP

201 KSFKLVQNKY LGVIIQCLVT ETKTSVSRHI YFFSARGRID PLVYLDEFLR

251 NSEPVLKRVN RTGNSSSNKQ EYQLLKDNLV RSYNKALKKN APYSIFAIKN

301 GPKSHIGRHL MTSFLSMKGL TELTNVVGNW SDKRASAVAR TTYTHQITAI

351 PDHYFALVSR YYAYDPISKE MIALKDETNP IEEWQHIEQL KGSAEGSIRY

401 PAWNGIISQE VLDYLSSYIN RRI

- FLP ENDS HERE -

424 SVRGS

- LINKER PEPTIDE ENDS HERE -

429 MK GGIRKDRRGG RMLKHKRQRD

451 DGEGRGEVGS AGDMRAANLW PSPLMIKRSK KNSLALSLTA DQMVSALLDA

501 EPPILYSEYD PTRPFSEASM MGLLTNLADR ELVHMINWAK RVPGFVDLTL

551 HDQVHLLECA WLEILMIGLV WRSMEHPVKL LFAPNLLLDR NQGKCVEGMV

601 EIFDMLLATS SRFRMMNLQG EEFVCLKSII LLNSGVYTFL SSTLKSLEEK

651 DHIHRVLDKI TDTLIHLMAK AGLTLQQQHQ RLAQLLLILS HIRHMSNKGM

701 EHLYSMKCKN VVPLYDLLLE MLDAHRLHAP TSRGGASVEE TDQSHLATAG

751 STSSHSLQKY YITGEAEGFP ATV

- ESTROGEN BINDING DOMAIN ENDS HERE -

Fig. 4/1

CODING SEQUENCE OF THE FLP/EBD FUSION PROTEIN ENCODED BY PHFE1

ATGCCACAATTTGATATATTATGTAAAACACCACCTAAGGTGCTTGTTCGT
CAGTTTGTGGAAAGGTTTGAAAGACCTTCAGGTGAGAAAATAGCATTATG
TGCTGCTGAACTAACCTATTTATGTTGGATGATTACACATAACGGAACAGC
AATCAAGAGAGCCACATTCATGAGCTATAATACTATCATAAGCAATTCGC
TGAGTTCCGATATTGTCAACAAGTCACTGCAGTTTAAATACAAGACGCAA
AAAGCAACAATTCTGGAAGCCTCATTAAAGAAATTGATTCCTGCTTGGGA
ATTTACAATTATTCCTTACTATGGACAAAAACATCAATCTGATATCACTG
ATATTGTAAGTAGTTTGCAATTACAGTTCGAATCATCGGAAGAAGCAGAT
AAGGGAAATAGCCACAGTAAAAAAATGCTTAAAGCACTTCTAAGTGAGG
GTGAAAGCATCTGGGAGATCACTGAGAAAATACTAAATTCGTTTGAGTAT
ACTTCGAGATTTACAAAAACAAAAACTTTATACCAATTCCTCTTCCTAGC
TACTTTCATCAATTGTGGAAGATTCAGCGATATTAAGAACGTTGATCCGA
AATCATTTAAATTAGTCCAAAATAAGTATCTGGGAGTAATAATCCAGTG
TTTAGTGACAGAGACAAAGACAAGCGTTAGTAGGCACATATACTTCTTTA
GCGCAAGGGGTAGGATCGATCCACTTGTATATTTGGATGAATTTTTGAGGA
ATTCTGAACCAGTCCTAAAACGAGTAAATAGGACCGGCAATTCTTCAAGC
AACAAGCAGGAATACCAATTATTAAAAGATAACTTAGTCAGATCGTACA
ACAAAGCTTTGAAGAAAAATGCGCCTTATTCAATCTTTGCTATAAAAAA
TGGCCCAAAATCTCACATTGGAAGACATTTGATGACCTCATTTCTTTCAAT
GAAGGGCCTAACGGAGTTGACTAATGTTGTGGGAAATTGGAGCGATAAGCG
TGCTTCTGCCGTGGCCAGGACAACGTATACTCATCAGATAACAGCAATACCT
GATCACTACTTCGCACTAGTTTCTCGGTACTATGCATATGATCCAATATCA
AAGGAAATGATAGCATTGAAGGATGAGACTAATCCAATTGAGGAGTGGC
AGCATATAGAACAGCTAAAGGGTAGTGCTGAAGGAAGCATACGATACCCC
GCATGGAATGGGATAATATCACAGGAGGTACTAGACTACCTTTCATCCTAC
ATAAATAGACGCATA

-FLP ENDS HERE-

TCCGTACGCGGATCC

- SYNTHETIC LINKER SEQUENCE ENDS HERE -

ATGAAAGGTGGGATACGAAAAGACCGAAGAGGAGGGAGAATGTTGAAAC
ACAAGCGCCAGAGAGATGATGGGGAGGGCAGGGGTGAAGTGGGGTCTGCTG
GAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGCTC
TAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCCT
TGTTGGATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAGACCC
TTCAGTGAAGCTTCGATGATGGGCTTACTGACCAACCTGGCAGACAGGGAGC
TGGTTCACATGATCAACTGGGCGAAGAGGGTGCCAGGCTTTGTGGATTTGAC

Fig. 4/2

CCTCCATGATCAGGTCCACCTTCTAGAATGTGCCTGGCTAGAGATCCTGATG
ATTGGTCTCGTCTGGCGCTCCATGGAGCACCCAGTGAAGCTACTGTTTGCTCCT
AACTTGCTCTTGGACAGGAACCAGGGAAAATGTGTAGAGGGCATGGTGGAG
ATCTTCGACATGCTGCTGGCTACATCATCTCGGTTCCGCATGATGAATCTGCA
GGGAGAGGAGTTTGTGTGCCTCAAATCTATTATTTTGCTTAATTCTGGAGTG
TACACATTTCTGTCCAGCACCCTGAAGTCTCTGGAAGAGAAGGACCATATCC
ACCGAGTCCTGGACAAGATCACAGACACTTTGATCCACCTGATGGCCAAGGC
AGGCCTGACCCTGCAGCAGCAGCACCAGCGGCTGGCCCAGCTCCTCCTCATCCT
CTCCCACATCAGGCACATGAGTAACAAAGGCATGGAGCATCTGTACAGCAT
GAAGTGCAAGAACGTGGTGCCCCTCTATGACCTGCTGCTGGAGATGCTGGAC
GCCCACCGCCTACATGCGCCCACTAGCCGTGGAGGGGCATCCGTGGAGGAGAC
GGACCAAAGCCACTTGGCCACTGCGGGCTCTACTTCATCGCATTCCTTGCAAA
AGTATTACATCACGGGGGAGGCAGAGGGTTTCCCTGCCACAGTCTGA

- HORMONE BINDING DOMAIN ENDS HERE -

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 94/02088

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07K15/04 C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE EMBO JOURNAL, vol.11, no.12, 1992, OXFORD pages 4641 - 4652 BOEHMELT ET AL. 'Hormone-regulated v-rel estrogen receptor fusion protein: ...' cited in the application see the whole document ---	1-6, 10-12, 16,17
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol.88, no.12, 15 June 1991, WASHINGTON, DC pages 5114 - 5118 SUPERTI-FURGA ET AL. 'Hormone-dependent transcriptional regulation .....' cited in the application see abstract ---	1-6, 10-12, 16,17

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
14 October 1994	28.11.94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ceder, O

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP 94/02088

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CELL, vol.54, 23 September 1988, CAMBRIDGE, MASS. pages 1073 - 1080 PICARD ET AL. 'A movable and regulable inactivation function .....' cited in the application see abstract ---	1
A	EP,A,0 146 050 (CALLEWAERT) 26 June 1985 see abstract ---	1
A	NUCLEIC ACIDS RESEARCH, vol.14, no.11, 1986, LONDON pages 4471 - 4481 CHEN ET AL. 'Sequence organization of the circular plasmid pKD1 ....' cited in the application see abstract ---	2-6,10
A	NUCLEIC ACIDS RESEARCH, vol.17, no.1, 1989, LONDON pages 147 - 161 SAUER ET AL. 'Cre-stimulated recombination ....' cited in the application see abstract ---	2-6,10
A	TRENDS IN GENETICS, vol.8, 1992, AMSTERDAM pages 432 - 439 STARK ET AL. 'Catalysis by site-specific recombinases' cited in the application -----	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 94/02088

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0146050	26-06-85	JP-A- 61122224	10-06-86
		CA-A- 1244361	08-11-88

Form PCT/ISA/210 (patent family annex) (July 1992)